

# Identification, Validation and Classification of the Genus *Phyllanthus* in Nigeria using ITS Genetic Marker and the Taxonomic Implication

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**Abstract:** An extraction, purification, PCR amplification and sequencing of DNA from five species of *Phyllanthus* in Nigeria namely *P. amarus* Schum and Thonn, *P. urinaria* Linn., *P. odontadenius* Mull-Arg., *P. niruroides* Mull-Arg. and *P. muellerianus* (O. Ktze) Excel belonging to the family of Phyllanthaceae were carried out using nuclear ribosomal Internal Transcribed Spacer (ITS 4-5) genetic marker to identify unknown *Phyllanthus* species. The nuclear region revealed that the *Phyllanthus* species were able to be amplified optimally for sequencing. The results of the nucleotide sequences were further compared on Basic Local Alignment Sequence Tool (BLAST) on GenBank and BoldSystems for validation. Results revealed that the closely related species, *P. niruroides* Mull-Arg. and *P. odontadenius* Mull-Arg. had no DNA record to separate them on both GenBank and BoldSystems while *P. amarus* Schum and Thonn, *P. muellerianus* (O. Ktze) Excel and *P. urinaria* Linn. were clearly compatible with other works. The sequence data were analyzed and classified with tree-based analyses of Mr.Bayes 3.2.1. in order to reveal their phylogenetic relationship. Results of the nucleotide sequences and fragment analysis were published on BoldSystems for barcoding as non-coding marker translation matrix.

**Keywords:** *Phyllanthus* species, DNA Extraction, PCR Amplification, Nucleotide Sequences, Gen Bank, Bold Systems, ITS, Molecular Phylogenetics

## 1. Introduction

Molecular phylogenetic studies have made important contributions to the classification of Phyllanthaceae since the work of Kathriarachchi *et al.* (2006). The molecular data of *Phyllanthus*, the third largest genus in the family, has received considerable attention as an important character in inferring systematic relationships (Wurdack *et al.*, 2004; Samuel *et al.*, 2005; Kathriarachchi *et al.*, 2005). Recent advances in understanding phylogenetic patterns of the pantropical family Phyllanthaceae (a segregate from Euphorbiaceae sensu lato [s.l.]) based on congruent plastid and nuclear DNA sequence data have recovered well-resolved and strongly supported clades (Wurdack *et al.*, 2004; Samuel *et al.*, 2005; Kathriarachchi *et al.*, 2005) that correspond to subfamilies and tribes.

*Phyllanthus* has a remarkable diversity of growth forms (annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous, and phyllocladous), floral morphology (Bancilhon, 1971), and chromosome numbers (Webster and Ellis, 1962). The diversity of pollen types (Köhler, 1965, 1967; Punt, 1967, 1987; Webster and Carpenter, 2002; Sagun and Van der Ham, 2003) rivals that of any genus of flowering plants. The vast majority of *Phyllanthus* species, however, share a distinctive vegetative specialization known as “phyllanthoid branching” (Webster, 1956) with leaves on the main axes reduced to scales called “cataphylls” and those on lateral (plagiotropic), deciduous, floriferous axes developing normally.

One of the most reliable methods for identification of

medicinal plants involves morphological and genetic analysis. Molecular techniques have been also introduced for DNA fingerprinting (Sucher and Charles, 2008). Analysis of the DNA that is present in all organisms is a suitable method for identifying plant materials because the genetic composition is unique for each individual organism. DNA extracted from the leaves, stems or roots of plants all carry the same genetic information without being affected by physiological conditions and environmental factors. Polymerase Chain Reaction (PCR) in combination with Sequencing and DNA barcoding has been widely used for DNA fingerprinting (Weisling *et al.*, 2005). Species-species regions in nuclear DNA, mitochondrial DNA and chloroplast DNA have been used for identification of individual species. The Internal Transcribed Spacer (ITS) region, found on either side of the transcribed region of the 18S, 5.8S and 26S nuclear ribosomal DNA, is one of the most popular sequences for phylogenetic analysis of plants for species-level identification (Sukrong *et al.*, 2007). The ITS region is highly repeated in the plant nuclear genome which is present in the form of up to many thousands copies arranged in tandem repeats. DNA fingerprinting is an important tool for molecular characterization of various groups of plants. It offers a faster and more precise way of determining relationships among closely related species than that of morphological investigation (Rahman, 2007). This is because morphological characteristics are subject to environmental influence and extensive studies of mature plants are often necessary for taxonomic classification. DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species (Paul *et al.*, 2003). It differs from molecular phylogeny in that the main goal is not to determine patterns of relationship but to identify an unknown sample in terms of a preexisting classification.

*Phyllanthus* has a long history of use in tropical countries in indigenous medicine for the treatment of liver ailment. They were examined during several researches. The pharmacognostic importance of some of these *Phyllanthus* species found in Nigeria has been elucidated by Awomukwu *et al.* (2014). These medicinal plants have been underutilized in orthodox medicine but have confirmed to be used worldwide in the pharmaceutical, food, cosmetics and

perfume industries. Different authors in different groups have studied the taxonomic significance and scientific implication of the morphological, anatomical and epidermal features of *Phyllanthus* species in Nigeria. They include Edeoga *et al.* (2007) and Uka *et al.* (2014). None of such works has successfully delineated the indigenous species occurring in Nigeria based on its DNA molecular data, sequencing and barcoding. Series of documented descriptions of the morphological, anatomical, ethnobotanical and phytochemical characteristics of *Phyllanthus* exist; there is confusion in recognizing individual species of these plants in Nigeria. The aim of this work is to provide reliable genetic information in order to aid easy recognition and to also understand the phylogenetic relationship among the *Phyllanthus* species for pharmacognostic research with reference to modern day genetic search tools.

## 2. Materials and Methods

### 2.1. Collection of Plant Materials

Mature plants of the five species *P. amarus*, *P. urinaria*, *P. niruroides*, *P. odontadenius* and *P. muellerianus* were collected from different locations of Nigeria (bounding box coordinates: upper left – 6.3333, 7; lower right – 4.75, 6.8333) by various investigators as in Table 1. Only healthy, fresh and succulent parts of the plants were collected. The five specimens were identified and authenticated at the Herbaria of the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State and the Department of Botany, Nnamdi Azikiwe University, Awka, Anambra State. Herbarium specimens were also studied at the various institutions as well making reference to the Flora of West Tropical Africa by Hutchinson and Dalziel (1963). The accessions were deposited at the Herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria with their sample and process identity numbers for further research. The same specimen records were also submitted on public data portal at Boldsystems. Further laboratory analysis was carried out at the Molecular Genetic Lab, University of the Free States, Bloemfontein, South Africa.

**Table 1.** Collection sites, identity numbers and collection dates of the five *Phyllanthus* species studied.

| SPECIES                | SITE OF COLLECTION  | COORDINATE/<br>ELEVATION | SAMPLE ID          | PROCESS ID  | DATE OF<br>COLLECTION |
|------------------------|---|--------------------------|--------------------|-------------|-----------------------|
| <i>P. amarus</i>       | Along the school fence, Abia State Polytechnic, Aba.            | N5.42; E6.33;<br>25.0m   | AWOM UPH PA<br>010 | PHSN 001-14 | April 15, 2014        |
| <i>P. urinaria</i>     | Field around National Root Crop Research Institute, Umudike.    | N4.75; E6.83;<br>20.0m   | AWOM UPH PN<br>050 | PHSN 003-14 | April 14, 2014        |
| <i>P. odontadenius</i> | Road side along National Root Crop Research Institute, Umuahia. | N5.42; E7.50;<br>25.0m   | AWOM UPH PO<br>040 | PHSN 006-14 | April 15, 2014        |
| <i>P. niruroides</i>   | Science Village, Nnamdi Azikiwe University, Awka.               | N6.33; E7.00;<br>22.0m   | AWOM UPH PU<br>030 | PHSN 005-14 | April 15, 2014        |
| <i>P. muellerianus</i> | Near the Herbarium Building, Nnamdi Azikiwe University, Awka.   | N6.33; E7.00;<br>23.0m   | AWOM UPH PM<br>020 | PHSN 002-14 | April 15, 2014        |

## 2.2. DNA Extraction and PCR Amplification

Total genomic DNAs were extracted from young, dry leaves of *Phyllanthus* specimens using the CTAB method by Doyle & Doyle (1987), because of high amount of polysaccharides present; the CTAB concentration was adapted to 3% (Doyle & Doyle 1990). The DNA pellet was then diluted into 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). An additional phenol/chloroform purification step was performed to remove proteins and potentially interfering secondary metabolites as follows: 150 µl of TE was added to bring the initial volume to 200 µl. Equal volume of phenol was then added, vortex gently and spin for 2 min at 13 000 rpm at room temperature. It was followed by addition of 100 µl of Sevag (chloroform-isoamyl alcohol 96:4), centrifugation at 13 000 rpm at room temperature of the collected aqueous steps (repeated twice). DNA was precipitated by incubating with 2/3 100% EtOH-3M NaOAc (25:1) at -20°C for 24 hours, then centrifuged for 2 min at 13 000 rpm at 4°C. The DNA pellet was washed with 70% EtOH, centrifuged at 13 000 rpm for 5 min, air dried in the dark and finally re-suspend in 50 µl TE.

The nuclear region ITS 4-5 (Table 2) was amplified using either direct PCR (Finnzymes Direct PCR kit) or iProof<sup>TM</sup> High-Fidelity DNA Polymerase (Bio-Rad Laboratories, CA) according to the manufacturer's protocol. iProof GC buffer (Bio-Rad) was used when nuclear phylogenetic gene

amplification with HF buffer (Bio-Rad) did not provide satisfactory results. A finally concentration of 5% DMSO was added in 20 µl PCR reaction. In few cases direct PCR was done on dry leaves or on fresh leaves stored at -20°C.

The PCR amplifications were performed using a G storm PCR system 9700 (Perkin-Elmer) with the following thermal cycle conditions unless stated otherwise: DNA was initially denatured at 98°C for 5 minutes followed by 40 cycles at 98°C for 5 seconds, primer annealing at 54.3°C for 5 seconds and elongation at 72°C for 20 seconds with a final 1 minute elongation at 72°C. The PCR fragment lengths were determined on a 1% agarose gel (see Fig. 1)

## 2.3. DNA Sequencing

PCR products were sequenced directly after 1:5 diluting with dH<sub>2</sub>O. Amplified regions were sequenced in both directions with an automated sequencer 3730 Genetic Analyser Applied Biosystems v1.1/3.1 Cycle Sequencing Kit, according to the protocol provided with few modifications. Briefly: the component and volumes for the sequencing PCR reactions were: 1 µl of 5x sequencing buffer, 0.5 µl premix (Applied Biosystems), 3 µl of 10 µM primer, 3 µl dH<sub>2</sub>O, 5% DMSO and 2 µl PCR product were used. Cycle sequencing steps were as follows: initial denaturation at 96°C for 1 min, following by 25x cycles of 96°C for 10 seconds (with a ramp seed of 3°Cs-1), 48°C for 15 seconds, 60°C for 4 min; 72°C for 1 min.

**Table 2.** Nucleotide sequence data of the primers used for the amplification of ITS regions.

| Region | Primer name | Primer sequence 5'-3' | Tm (°C) | MW     | GC(%) | Length |
|--------|-------------|-----------------------|---------|--------|-------|--------|
| ITS    | ITS 5       | GGAAGTAAAAGTCGTAACAAG | 51.3    | 6865.5 | 38.1  | 21     |
|        | ITS 4       | TCCTCCGCTTATTGATATGC  | 52.1    | 6034.0 | 45.0  | 20     |

## 2.4. Clean up

Extension products were adjusted to 20 µl by adding 10 µl of dH<sub>2</sub>O, precipitated with 5 µl of 125 mM EDTA, and 60 µl of absolute EtOH for 15 min and centrifuged for 15 min at 20 000 g. For pellet purification, 60 µl of 70% EtOH was added after removal of supernatant, centrifuged for 5 min at 20 000 g at 4°C. Tubes were then dried at 55°C for 5 min.

## 2.5. Sequence Alignment and Data Analysis

The data sequences of ITS region were aligned, followed by manual adjustment and trimming at the ambiguous ends. The software programme Geneious Pro 7.0.4 (Biomatters, Ltd., <http://www.geneious.com>) with the default alignment parameters was used to view, assemble and edit the sequence trace files. Consensus sequences were aligned with the MUSCLE plug-in in Geneious R7. The post-trimmed lengths were at least 80% of the original read length. Sequences which covered more than 70% overlap between the forward and reverse sequences were considered. Other statistical values for each gene region such as the composition, GC content, % pairwise residue, % identical alignment, the minimum, maximum and mean lengths for the sequences as well as the pairwise number and percentage identity, identities and

differences of bases/ residues were also obtained in Geneious R7. Consensus sequences were compared on GenBank nucleotide Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information website ([www.ncbi.nlm.nih.gov/Blast.cgi](http://www.ncbi.nlm.nih.gov/Blast.cgi)) and Boldsystems (<http://www.boldsystems.org>) for species identity authentication.

Character-based tree drawing method: The MrBayes 3.2.1 (Huelsenbeck & Ronquist, 2001) plugin to Geneious R7 was used to construct a dendrogram which are diagrams of the phylogenetic relationships between the taxa using their genetic information. Pairwise differences were used to cluster the samples in Molecular Operational Taxonomic Units using the program. Phylogenetic analysis was performed using the maximum parsimony (MP) and maximum likelihood (ML) character-based. All the characters were equally weighed and unordered with the highest bootstrap support for a monophyletic group.

## 2.6. DNA Barcoding Method

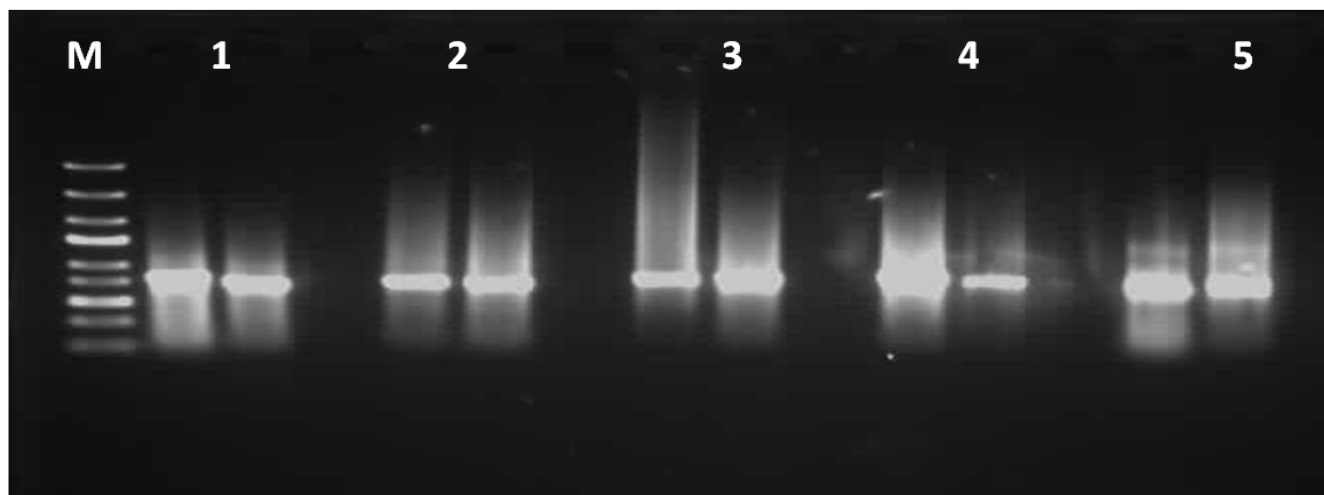
The project was registered under the workbench portal on the website (<http://www.boldsystems.org>). Information on the title, code, description, campaign and markers were provided. Then after, the details of the specimen data (such as species

name, voucher data, collection record, identifier and primer information), images, sequences and trace files were uploaded by following the formal submission guidelines and instructions on the website. The deposited sequences are automatically tested for incongruence by the website software and validated by the database administrators before publication.

### 3. Result

In this study, DNA quality was assayed by gel

electrophoresis and intense bands were seen on 0.8% agarose. Only duplicate samples of each species were selected for sequencing (Plate 1). The thick band shows the PCR amplified ITS region which ranged from 500bp – 600bp in the respective samples. The leaf materials used were mainly dry leaves stored in silica gel. The samples contained both old and young leaves. The older and harder leaves had a higher level of secondary compounds which could act as PCR inhibitory components. The young leaves of *Phyllanthus* yielded a better quality DNA than the older, outer leaves of the plants.



**Plate 1.** PCR profile of the amplified ITS gene region of the leaf of the five *Phyllanthus* species investigated. DNA fragment size range from 500bp – 600bp. Each species is represented by a duplicate of the samples. 1 – *P. amarus*, 2 – *P. muellerianus*, 3 – *P. niruroides*, 4 – *P. odontadenius*, 5 – *P. urinaria*.

When visualised on an agarose gel, the DNA from the young leaves had less or no smears. Even by adapting the extraction protocol by adding recommended chemicals (such as PVP and SDS) at different stages in the process, the secondary compounds (e.g. polysaccharides and polyphenolic compounds) could not be removed completely. The polysaccharides co-precipitate with the DNA (Kumari *et al.*, 2012), resulting in a viscous solution. Some leaf samples, where the direct PCR approach was followed, seemed to contain PCR inhibitory components, and were excluded from the study after 3-4 amplification attempts. For future studies

on *Phyllanthus* it is suggested that the young leaves are used for DNA extraction, or alternatively that the method of Kumari *et al.* (2012) be implemented as an extraction protocol.

The aligned ITS matrix consisted of 541 base pairs (bp). The maximum parsimony of the ITS region produced, 83.4% pairwise residue, 69.6% identical alignment, 534 bp maximum sequence and 517 bp minimum sequences (Table 3). The strict consensus tree with bootstrap percentages is shown in Fig. 1.

**Table 3.** Matrix and tree statistics of the ITS data sets in the study.

| Sampling Information        | ITS Analysis | Description   |
|-----------------------------|--------------|---|
| Sequence Length (bp)        | 541          | Residue length of sequence.   |
| Number of species sequences | 5            | Number of sequences in an alignment or set of sequences.  |
| Pairwise residue (%)        | 83.4         | Percentage of pairwise residues that is identical in the alignment, including gap vs non-gap residue, but excluding gap vs gap residue. |
| Identical alignment (%)     | 69.6         | Percentage of columns in the alignment for which all sequences are identical.   |
| Maximum sequence (bp)       | 535          | Maximum length of non-reference sequences in a sequence list or alignment.  |
| Minimum sequence (bp)       | 518          | Minimum length of non-reference sequences in sequence list or alignment.  |

The differences in the number of bases/residues which are not identical (Table 4) and identities of number of bases/residues which are identical (Table 5) obtained in these five *Phyllanthus* species showed a distinct variation.

Similarity matrix which shows the percentage identity distances (Table 6) was generated from the Geneious R7 software during the analysis.

**Table 4.** Differences – Number of bases/residues which are not identical (bp).

| TAXA                   | <i>P. amarus</i> | <i>P. muellerianus</i> | <i>P. urinaria</i> | <i>P. odontadenius</i> | <i>P. niruroides</i> |
|------------------------|------------------|------------------------|--------------------|------------------------|----------------------|
| <i>P. amarus</i>       | -                | 109                    | 105                | 106                    | 105                  |
| <i>P. muellerianus</i> | 109              | -                      | 117                | 113                    | 112                  |
| <i>P. urinaria</i>     | 105              | 117                    | -                  | 84                     | 82                   |
| <i>P. odontadenius</i> | 106              | 113                    | 84                 | -                      | 17                   |
| <i>P. niruroides</i>   | 105              | 112                    | 82                 | 17                     | -                    |

**Table 5.** Identities – Number of bases/residues which are identical (bp).

| TAXA                   | <i>P. amarus</i> | <i>P. muellerianus</i> | <i>P. urinaria</i> | <i>P. odontadenius</i> | <i>P. niruroides</i> |
|------------------------|------------------|------------------------|--------------------|------------------------|----------------------|
| <i>P. amarus</i>       | -                | 425                    | 423                | 423                    | 423                  |
| <i>P. muellerianus</i> | 425              | -                      | 419                | 424                    | 424                  |
| <i>P. urinaria</i>     | 423              | 419                    | -                  | 444                    | 444                  |
| <i>P. odontadenius</i> | 423              | 424                    | 444                | -                      | 510                  |
| <i>P. niruroides</i>   | 423              | 424                    | 444                | 510                    | -                    |

The percentage identity (Table 6) was generated from the analysis of the tree builder plug-in in Geneious R7 software by calculating the differences between the sequence length

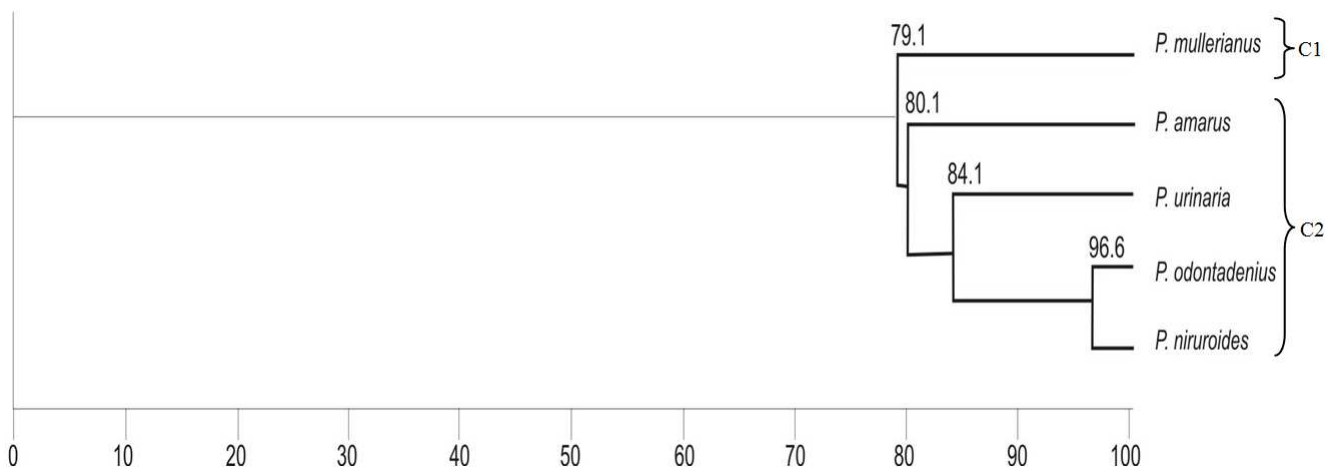
and number of bases/residues which are not identical within each species, divided by the sequence length and multiplied by hundred.

**Table 6.** % Identity – Percentage of bases/residues which are identical.

| TAXA                   | <i>P. amarus</i> | <i>P. muellerianus</i> | <i>P. urinaria</i> | <i>P. odontadenius</i> | <i>P. niruroides</i> |
|------------------------|------------------|------------------------|--------------------|------------------------|----------------------|
| <i>P. amarus</i>       | -                | 79.6                   | 80.1               | 80.0                   | 80.1                 |
| <i>P. muellerianus</i> | 79.6             | -                      | 78.2               | 79.0                   | 79.1                 |
| <i>P. urinaria</i>     | 80.1             | 78.2                   | -                  | 84.4                   | 84.1                 |
| <i>P. odontadenius</i> | 80.0             | 79.0                   | 84.4               | -                      | 96.6                 |
| <i>P. niruroides</i>   | 80.1             | 79.1                   | 84.1               | 96.6                   | -                    |

The dendrogram (Fig 1) generated from MrBayes pluggin in Geneious R7 revealed the molecular phylogeny of the five accessions being analyzed. From the result, *P. odontadenius* and *P. niruroides* showed the closest similarity at 96.6% in the ITS genome region while *P. amarus* and *P. muellerianus*

displayed the least similarity (79.1%) when compared with other taxa in the study. The dendrogram comprises of two clusters C1 and C2 in which cluster C1 is distinctly *P. muellerianus* while cluster C2 comprises of *P. amarus*, *P. urinaria*, *P. odontadenius* and *P. niruroides*.

**Fig. 1.** Dendrogram of the molecular phylogeny of the five *Phyllanthus* species.

During the research in BOLD, the different sequences were compared and the differences were represented in a form that resembles barcodes in which each colour represents one of the nucleotide bases of DNA [A, T, C or G]. Like a barcode for products in a shop, each species of the *Phyllanthus* investigated had unique nucleotide barcoding pattern in BOLD and were identifiable from this unique

pattern. Other *Phyllanthus* barcodes submitted on BOLD were compared with our accessions and only *P. amarus* and *P. urinaria* had previous records of other authors. *P. muellerianus*, *P. niruroides* and *P. odontadenius* had no previous submitted sequence records on BOLD and therefore were the first time to be registered and submitted on the website (<http://www.boldsystems.org>). Table 7 displays the

information on the link of the accessions published on BOLD.

**Table 7.** Information and links of the *Phyllanthus* species accessions submitted on BOLD Systems.

| TAXA                   | PROCESS ID  | SEQUENCE ID     | LINK/REFERENCE  |
|------------------------|-------------|-----------------|---|
| <i>P. amarus</i>       | PHSN 001-14 | PHSN 001-14.ITS | <a href="http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN001-14">http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN001-14</a> |
| <i>P. muellerianus</i> | PHSN 002-14 | PHSN 002-14.ITS | <a href="http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN002-14">http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN002-14</a> |
| <i>P. niruroides</i>   | PHSN 005-14 | PHSN 005-14.ITS | <a href="http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN005-14">http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN005-14</a> |
| <i>P. odontadenius</i> | PHSN 006-14 | PHSN 006-14.ITS | <a href="http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN006-14">http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN006-14</a> |
| <i>P. urinaria</i>     | PHSN 003-14 | PHSN 003-14.ITS | <a href="http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN003-14">http://www.boldsystems.org/index.php/Public_RecordView?processid=PHSN003-14</a> |

## 4. Discussion

The identification of *Phyllanthus* species using the sequences of the nuclear ribosomal Internal Transcribed Spacer (ITS) genetic marker is seen as a promising tool for the authentication plant species and ensuring better quality herbs and pharmaceuticals. This is otherwise known as DNA fingerprinting apart from identifying alterations in the genotypes of plant species, is also used for the betterment of drug-yield by tissue culturing and of which *Phyllanthus* is certainly one of the most useful herbs in the world.

According to Henry (2001), DNA of interest can be stored as germplasm, which is then used for future cultivation. In addition, germplasm can be used for the conservation of selected plant species, which are endangered. Although morphology, anatomy and phytochemistry can be used as useful tools in biosystematics but factors such as soil, climate and adaptability dictate the viability of a particular species and subsequently its contents but such discrepancies cannot be tolerated by DNA fingerprinting technique. DNA fingerprinting has been found to be useful in identifying species, which may have morphological or anatomical difficulties.

The close morphological similarities of members of the genus *Phyllanthus* in Nigeria often times lead to confusion in species identification and collection. Existing taxonomic on the genus in the country is based on morphological characters documented in Hutchinson and Dalziel (1963), which reported some indeterminate species. Reassessment of the phylogenetic relationship of the members of this genus is therefore worthwhile. We have begun this by subjecting species within the southern region of the country to DNA characterization, numerical analysis and phylogenetic interpretation.

Dendrogram based on the DNA information of the ITS genome region grouped these five southern species into two major clusters C1 and C2. Cluster C1 is distinct containing only *P. muellerianus*. This indicates that *P. muellerianus* is distinct from all other southern species studied. The remaining four species clustered in C2. This also agrees with morphological characteristics of *P. muellerianus* as documented by Hutchinson and Dalziel (1963). The habit of *P. muellerianus* is tree while others are herbs conforming to the molecular similarity matrix in having the farthest distance of 79.1% when compared with other species. In cluster C2, *P. amarus* is distinct among the other species in this cluster. *P. niruroides* and *P. odontadenius* are closely related (96.9%) although this does not support the morphological features

between them when compared with the floral morphology. *P. niruroides* possess five sepals while *P. odontadenius* possesses six sepals as documented by Hutchinson and Dalziel (1963) although they both share similar foliar morphology and habit. Similarly *P. muellerianus* and *P. odontadenius* are the most diverged from the result (Table 6) and in the dendrogram (79.0%). On the other hand, *P. urinaria* is closely related to *P. niruroides* and *P. odontadenius* at 84.1% and 84.4% similarity respectively (Table 6). From dendrogram, it shows that *P. niruroides* and *P. odontadenius* probably share ancestry lineage with *P. urinaria* (Fig 1). All clades are monophyletic because they arise from a common ancestor. From the dendrogram, it revealed that other species actually arose from *P. muellerianus* before divergence of other species began.

It is important to reemphasize the fact that current information is lacking on members of the genus *Phyllanthus* in Nigeria. These results indicate that ITS is a good barcoding candidate but on its own, it is insufficient to identify all the species. The efficiency of a two-barcode should be investigated but due to the size of the genus, it is likely that a three or more gene barcode will be necessary. Although several loci have been suggested, a common set of standardized regions were selected by the respective communities in order to provide a large variation between species yet a relatively small amount of variation within a species. In BoldSystems, the concatenation of the *rbcl* and *matK* chloroplast genes had always been the desirable choice of locus (Paul *et al.*, 2003).

Currently in Nigeria, there is need for taxonomists to employ the system of molecular identification of every plant in order to establish a standard genetic library. This will help to curtail the confusion created by ambiguous morphological identification. Moreover, it will also aid to review obsolete literature in the field of taxonomy because recent discoveries will eradicate wrong information on overlapping species identification, nomenclature and classification.

## 5. Conclusion

The genus *Phyllanthus* remains an interesting and challenging genus. This study has proved to us that while we try and fit all species in its own containers, nature is alive, has its own way and is constantly developing and changing. We, humans have a huge need to identify, describe and name species. Maybe one day we will be able to do it our own satisfaction, but in the meantime, we have this gift to humanity called the *Phyllanthus* – that is there for us to enjoy and study.

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